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IMPROVED PROCEDURES FOR HUMAN GRANULOSA CELL ISOLATION AND LONG-TERM IN VITRO EXPANSION

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Abstract

Granulosa cells represent an essential part of the ovary's somatic compartment that maintains oocyte development and maturation and also provides the hormonal environment necessary to coordinate uterine receptivity with ovulation and, subsequently, to sustain early pregnancy. Primarily due to their major role in the processes of steroidogenesis and folliculogenesis, the study of granulosa cells is likely to be essential for comprehending and elucidating the cellular and molecular mechanisms underlying ovarian development and functions in both physiological and pathological conditions. Density gradient centrifugation is the primary method for the isolation of these cells from follicular fluid. However, this technique is time-consuming and in demand of additional reagents, experience, and dexterity. Furthermore, the limited half-life and proliferative potential of human granulosa cells put additional challenges for their study, making the conventional approaches for cultivation less applicable.

Our aim was to develop dependable and effective protocols for the isolation and long-term cultivation of primary granulosa cells while preserving their functional and morphological characteristics.

Human granulosa cells were isolated from follicular fluid by centrifugation and treatment with hyaluronidase without using density gradient centrifugation and/or red blood cell lysis buffer procedures. Steroidogenesis, growth

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dynamics, and the population doubling time of granulosa cells cultivated in a newly designed expansion medium, were determined. The medium consisted of DMEM/F12 supplemented with 5% human serum and 33% human follicular fluid.

The isolation technique described in this study ensures the generation of granulosa cells with a very high level of purity. Granulosa cells cultivated in the newly developed expansion medium actively grew without changing morphologically and without losing the ability to produce estradiol and progesterone. Moreover, in this particular expansion medium, granulosa cells demonstrated a significantly increased proliferation rate, with a much shorter population doubling time compared to the control cultures grown in medium containing fetal bovine serum.

Here we present a simple, fast, and cost-effective procedure for human granulosa cell isolation, combined with a reliable approach for their rapid growth and long-term cultivation based on a newly formulated expansion medium. Together, these two methods provide a comprehensive system for generating granulosa cell cultures, enabling their extensive study for the purposes of reproductive biology and medicine.

Key words: human granulosa cells, isolation, long-term cultivation, optimized procedures, human serum, follicular fluid

Introduction. Granulosa cells (GCs) are a vital part of the somatic compartment of the ovary and play a central role in regulating follicular development. The formation of a mature and developmentally competent oocyte is a complex process involving a close interaction between germ and somatic cells [1]. In this context, GCs are involved in a two-way metabolic communication through gap junctions with the oocyte, a process essential for the proper maturation of the oocyte and the functionality of granulosa cells [2]. On the other hand, the second primary function of GCs is the production of the key sex steroids estradiol and progesterone, which vary in concentration depending on the stage of the menstrual cycle [1]. During the follicular phase, GCs, under the stimulation of follicle-stimulating hormone (FSH), convert androgens from theca cells into estradiol via cytochrome P450 aromatase. Estradiol secretion is crucial for the proliferation and maintenance of the endometrial lining, regulation of the hypothalamic-pituitary-ovarian axis, and the induction of the luteinizing hormone (LH) surge required for ovulation. After ovulation, during the luteal phase, GCs transform into luteinized granulosa cells, producing progesterone, which is essential for maintaining the uterine lining and supporting a possible pregnancy [3, 4].

Due to their pivotal role in steroidogenesis and folliculogenesis, studying granulosa cells is crucial for understanding the largely unknown cellular and molecular mechanisms that govern ovarian development and functions in both normal and pathological conditions. A typical source of freshly harvested human GCs for basic research is follicular fluid collected during in vitro fertilization (IVF) procedures. The main technique used to isolate these cells from human follicular fluid

involves density gradient centrifugation (DGC), employing colloidal products such as Percoll or Ficoll [5,6]. However, this technique is time-consuming and requires specialized reagents and expertise. Moreover, the colloidal particles are not entirely biologically inert and pose a risk of inducing cellular activation during the isolation and cultivation [7]. Because of their terminally differentiated state, human GCs also have inherent limitations for long-term in vitro studies, including a short life span and limited proliferative capacity. As a result, conventional culture methods using standard media supplemented with fetal bovine serum (FBS) often fail to support their growth effectively [8]. However, the preparation of specialized basal culture media to address these challenges involves the inclusion of numerous supplements, growth factors, and hormones, which not only significantly increase costs but also frequently lead to suboptimal results [9,10].

In light of these challenges, our goal was to develop dependable, efficient, and cost-effective procedures for the isolation and prolonged cultivation of primary GCs. At the same time, our approach aims to preserve their functional integrity and morphological characteristics, enabling more effective and sustainable use in research. Overcoming the limitations of conventional methods will contribute to the advancement of studies on granulosa cells and their applications in reproductive biology and its related fields.

Materials and methods. ***Ethics statement.*** All participants signed informed consent for collection and use of their otherwise discarded follicular fluid/granulosa cells for research purposes. The study and consent form were approved by the Ethics Committee of Ob/Gyn Hospital Dr. Shterev (Sofia, Bulgaria).

Follicular fluid collection. Human primary granulosa cells were obtained from pre-ovulatory follicles of 30 women undergoing oocyte retrieval in the course of an IVF procedure at the Ob/Gyn Hospital Dr. Shterev, Sofia. Following the removal of cumulus-oocyte complexes from the follicular aspirates, the remaining follicular fluid (FF) from each patient containing granulosa cells was pooled in sterile 15 ml conical polypropylene tubes (EuroClone) and processed within an hour. Samples that were significantly contaminated with erythrocytes were not included in the study.

Granulosa cell isolation and cultivation. The follicular aspirates were centrifuged at 300 × g for 10 min, after which the supernatant consisting of cell-free follicular fluid was collected and stored at −20 °C for further use as a component of the granulosa cell cultivation medium. The cell pellet was resuspended in a hyaluronidase solution (25 IU; SynVitro® Hyadase, CooperSurgical) and incubated for 3 min with continuous shaking. Following the addition of 10 ml of DMEM-F12 (D8062, Sigma-Aldrich) supplemented with 10% (v/v) human serum (HS; H4522, Sigma-Aldrich) and 1% (v/v) antibiotic/antimycotic solution (100×; A5955, Sigma-Aldrich), a centrifugation was performed at 300× g for 10 min. The pelleted cells were then resuspended, seeded, and grown in a newly de-

signed expansion medium consisting of DMEM-F12 supplemented with 5% (v/v) human serum, 1% (v/v) antibiotic/antimycotic solution, and 33% (v/v) human follicular fluid. Control cultures were maintained in low-glucose (1.0 g/L) DMEM (DMEM-LG; D6046, Sigma-Aldrich) containing 10% (v/v) fetal bovine serum (FBS; F7524, Sigma-Aldrich) and 1% antibiotic/antimycotic solution. Isolated cells were plated in 10 cm^2 6-well plates (EuroClone) and were incubated under standard culture conditions (humidified atmosphere, 37 °C, 5% CO_2), with the cell culture media being changed every 3 days. On reaching confluence, cells were harvested (0.25% trypsin/0.02% EDTA; T4049, Sigma-Aldrich) and expanded in 25/75 cm^2 flasks (EuroClone) or used for further experiments.

The morphological status of the cells in each passage was assessed through direct observation of live cultures under an inverted light microscope (Leica DMI3000 B), with images captured and analyzed using a digital camera (Leica DMC2900) and associated Leica Application Suite (LAS) software.

Flow cytometry analysis. Cultured human granulosa cells at 2nd passage were harvested (0.25% trypsin/0.02% EDTA), washed with phosphate buffered saline (PBS), and sequentially fixed in 4% ice cold and freshly prepared buffered paraformaldehyde (20 min; room temperature), washed with PBS, and permeabilized with 0.1% Triton X-100 for 60 min at 4 °C. After two further washes, samples containing 1×10^5 cells were prepared and incubated for 60 min at 4 °C with specific anti-human monoclonal anti-LHR (luteinizing hormone receptor; 8G9A2, MA5-31793, Thermo Fisher Scientific) and anti-FSHR (follicle-stimulating hormone receptor; 3D5G9, MA5-38525, Thermo Fisher Scientific) antibodies. After two washing steps in PBS, secondary goat anti-mouse IgG1 Alexa Fluor® 488 (A-21121, Thermo Fisher Scientific) was added and samples were incubated for 30 min at 4 °C. For aromatase cytochrome P450 (CYP19A) detection, samples were directly incubated for 30 min (4 °C) with anti-human fluorochrome-labelled monoclonal antibody anti-CYP19 (E-9) Alexa Fluor® 647 (sc-374176 AF647; Santa Cruz). Cells unlabelled with antibodies or isotype controls were used as autofluorescent controls. The specific fluorescence staining was analyzed on a FACS Calibur flow cytometer (BD Biosciences) with 100 000 events, and the results were processed using the Cell Quest software (BD Biosciences).

Cell proliferation and population doubling time (PDT). Human granulosa cells from the 2nd to 8th passages were seeded in quintuplicates on 96-well plates (EuroClone) at a density of 2.5×10^3 cells/well and cultured for 8 days in the two distinct expansion media previously described. AlamarBlue (AB) assay was performed to assess the cellular growth. Cell proliferation was evaluated every 48 h by measuring the reduction of alamarBlue fluorometric/colourimetric growth indicator according to the manufacturer's instructions (alamarBlue®, Bio-Rad Laboratories). The growth medium was replaced with 1:10 diluted AB stock solution in the appropriate culture medium and cells were incubated for 4 h at 37 °C and 5% CO_2. Then, 100 µl aliquots were transferred to a fluoro-microtitre plate

(Costar, Corning Incorporated) and fluorescence was measured at wavelengths of 544 nm (excitation) and 590 nm (emission) on a FLUOstar OPTIMA microplate reader (BMG Labtech). As this assay is non-toxic to cells, the culture medium was replaced with a fresh one after each measurement, ensuring the maintenance of the same cultures until the end of the experimental period. A standard curve was made by seeding a different number of cells in 96-well plate (EuroClone) and AB fluorescence intensity was evaluated as described.

The population doubling time (PDT) was calculated using the formula:

$$PDT = \frac{(t - t_0) \times \log(2)}{\log(C) - \log(C_0)},$$

where $t - t_0$ is the culture time (h); C is the final cell concentration; and C_0 is the initial cell concentration.

Steroid hormone assay. To evaluate the estradiol and progesterone production, human granulosa cells from the 2nd to 8th passages were seeded on 24-well plates (EuroClone) at a density of 2.0×10^4 cells/well in DMEM/F12 supplemented with 5% (v/v) human serum and 1% (v/v) antibiotic/antimycotic solution. After 48 h of cultivation at 37 °C, 5% CO_2, cell culture media were collected, centrifuged, and stored at −80 °C until analysis. The levels of secreted 17β-estradiol and progesterone in the conditioned media were measured by an electrochemiluminescence immunoassay (ECLIA; Elecsys Estradiol III, ref. 06656021190; Elecsys Progesterone III, ref. 07092539190; Cobas, Roche Diagnostics) on the Roche Elecsys 2010 Immunoassay Analyzer (Roche Diagnostics). All analyses were carried out in triplicate, and mean values were presented.

Data analysis and statistics. The Student's t-test or non-parametric Mann–Whitney U rank sum test was applied to evaluate the statistical significance of the difference between mean values. These statistical analyses were carried out using SigmaPlot software (v12.5, Systat Software, Inc.), and the results are expressed in the text and figures as mean values ± standard deviation (SD). Regression analyses were performed using CurveExpert Basic (version 1.4, Hyams Development) statistical software. For all analyses, differences were considered statistically significant at p-value ≤ 0.05.

Results. Cell isolation from follicular fluid using the procedure described in "Materials and methods" provided a significant initial quantity of granulosa cells. Small cell aggregates and scattered single adherent cells were observed on the bottom of the culture vessel 24 h after their initial seeding (Fig. 1A). The isolated adherent cells varied both in size and shape, exhibiting elongated or irregular morphology, and had a relatively big nucleus and granulated cytoplasm (Fig. 1). Any erythrocytes that might have contaminated the primary cell culture were completely removed during media changes and culture harvesting.

Through this method, human granulosa cells were successfully isolated from the follicular fluid samples of all 30 donors included in the study.

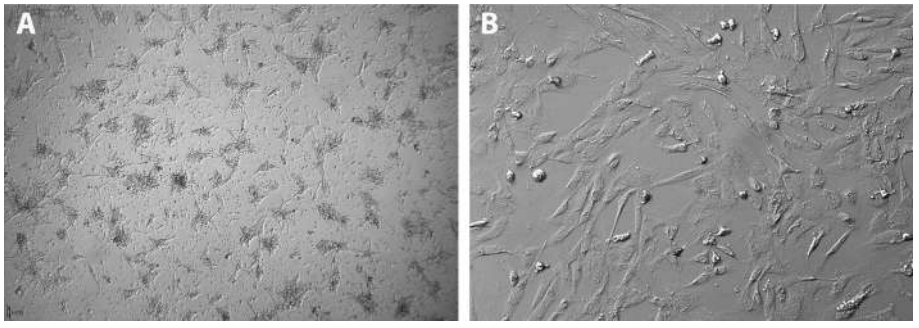

Fig. 1. Morphological characteristics of human granulosa cells after 24 h of initial culture (magnification, × 60) **(A)** and at passage 6 (magnification, × 100) **(B)**

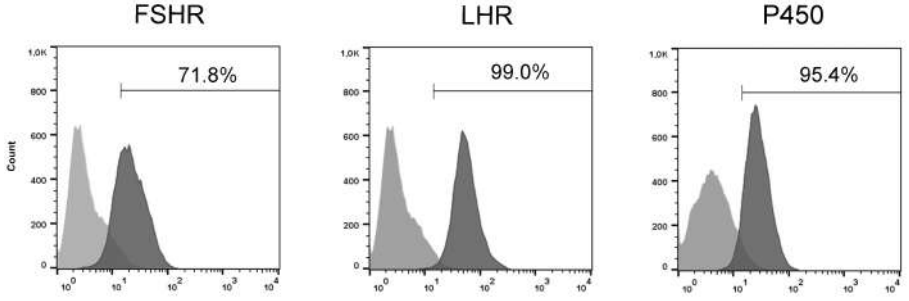

Fig. 2. Immunophenotypic profile of newly isolated human granulosa cells

The isolated ovarian cells were characterized as GCs through their marker expression profile and the steroid hormone production. Flow cytometric analysis showed that the vast majority of the cells at passage 2 were positive for FSHR (71.8%), LHR (99.0%), and aromatase cytochrome P450 (95.4%; Fig. 2). Furthermore, we found out that the cell culture-conditioned media from all the cultures tested contained estradiol and progesterone, with mean concentration values of 2790 ± 650.2 pmol/L and 229 ± 67.7 nmol/L, respectively.

As shown in Fig. 3A, GCs cultures maintained in DMEM-F12/HS/FF proliferated significantly faster than those cultured in DMEM-LG/FBS. According to the results, the average population doubling time (PDT) of the GCs grown in the newly developed expansion medium was 66.5 hours (Fig. 3B), which is 3.8 times shorter than that of the DMEM-LG/FBS controls (253.6 hours, $p < 0.01$; Fig. 3B). GCs cultivated in DMEM-F12/HS/FF maintained their high proliferation rates at least until passage 8, retaining their morphological characteristics and the ability to produce estradiol and progesterone. In contrast, control DMEM-LG/FBS cultures ceased to grow and died after passage 4.

Discussion. The presented research had the goal of developing optimized, reliable, and efficient protocols of isolation and cultivation of human GCs that may overcome some limitations of the currently widely used methods. The results show

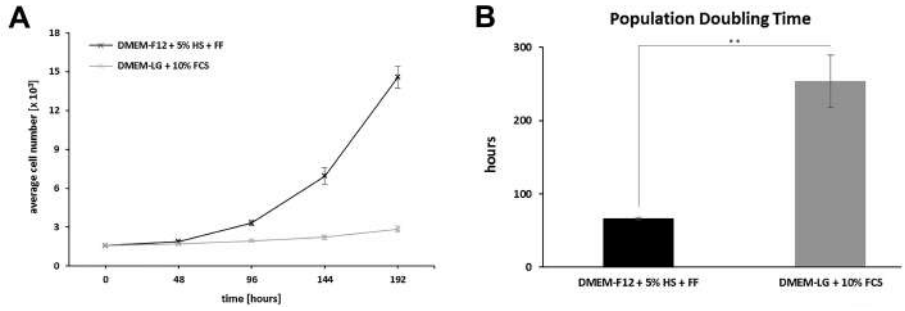

Fig. 3. Proliferation profiles **(A)** and average population doubling times **(B)** of human granulosa cells cultured in DMEM-F12/HS/FF (■) or DMEM-LG/FBS (■; control cultures). The data are presented as mean ± SD, $^{**}p < 0.01$

that GCs have been successfully isolated and cultivated for an extended period of time while maintaining their steroidogenic potential and functional integrity.

In the current study, human GCs were isolated from the follicular fluid by centrifugation and hyaluronidase treatment without density gradient centrifugation and/or red blood cell lysis buffer (RLB) procedures. This is a relatively quicker, easier, and more convenient approach that avoids supplementary reagents, hence saving costs. The morphological characteristics and marker expression profiles of isolated cells confirmed their granulosa cell identity and demonstrated that the described isolation technique generates a primary culture of granulosa cells with a very high degree of purity. Namely, the majority of isolated cells expressed key phenotypic markers of GCs, such as FSHR, LHR, and aromatase cytochrome P450 [11]. Further, we have demonstrated another key feature of GCs, specifically their ability to produce estradiol and progesterone, underlining their functional completeness and suitability for studies on ovarian physiology and steroidogenesis.

The increased proliferation rates and extended culture life span of GCs in the presence of human serum and follicular fluid represent significant improvements over conventional culture conditions in FBS-supplemented medium. It has been shown [8] that when isolated human GCs were cultivated in vitro under conventional conditions using FBS-supplemented media, the culture grew slowly, lasting no more than four passages, with a population doubling time of approximately two weeks (336 hours). These data are very similar to our findings concerning the control DMEM-LG/FBS cultures, which ceased growing and died after passage 4, having a PDT of 253.6 hours.

The newly formulated medium decreased the population doubling time (PDT) 3.8-fold over the DMEM-LG/FBS controls. Rapid proliferation, maintained up to at least 8 passages, providing a robust system for generation of adequate cell quantities for experimental studies. Moreover, the fact that steroid production and morphological integrity were maintained through passages indicated that the medium was capable of supporting GC functions over long-term culture.

The supplementation of the medium with follicular fluid offers a distinct advantage by providing an in vivo-like physiological environment for GCs. Follicular fluid contains a complex mixture of growth factors, hormones, and cytokines [12] important for GC functions and oocyte maturation. This could be a contributing factor to the higher proliferation rates and the maintenance of steroidogenic activity, as seen in our study. This again stresses the importance of the re-establishment of the native microenvironment for the support of cellular functions in vitro. Moreover, human serum has been used instead of fetal bovine serum, adding even more advantages. Human serum more closely resembles the natural extracellular environment, further enhancing the physiological compatibility of the culture conditions. Especially, HS supplementation is beneficial to human granulosa cells as it may contain species-specific components that promote more effectively cell proliferation and differentiation compared to FBS. Avoidance of the use of FBS also eliminates ethical issues and discrepancies related to the use of animal-derived products and, in this context, provides more standardized and clinically relevant culture system.

Overall, it is evident that the translational potential of this optimized GC culture system is significant, while traditional protocols for reliable and efficient GC models are invaluable in the study of ovarian pathologies including polycystic ovary syndrome, endometriosis, and premature ovarian insufficiency. Moreover, these improved methods can be used in toxicological testing, drug development, and understanding the molecular basis of infertility. The long-term culture of GCs also provides opportunities to explore long-term cellular and molecular responses to experimental interventions.

In summary, the protocols developed herein surmount major limitations of traditional methods for the isolation and cultivation of GCs by providing a simpler, faster, less expensive, and more efficient approach. This methodology ensures the functional and morphological characteristics of GCs over multiple passages, thus providing a solid platform for advancing research in ovarian biology and reproductive medicine.

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